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Mini Review

Challenges, Opportunities and Best Practice when Using miRNA as Disease Biomarkers in Liquid Biopsies

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Abstract

Early diagnosis and monitoring of treatment are essential for the efficient management of patients. In this regard, significant efforts have been made to find informative, blood (and other biological fluids) -based biomarkers. Thanks to their innate characteristics, microRNAs (miRNAs) hold the promise of being ideal biomarker molecules for healthcare needs and in cancer in particular. Consequently, the number of studies exploring such exciting possibility has multiplied exponentially in the last couple of years. However, the caveat is that this field is still very much in its infancy, and special attention must be paid to the technological aspects involved in miRNA handling and detection, if miRNAs are to fully realize their potential. In this mini review, we outline the clear possibilities of the circulating miRNome as a source of clinically-relevant biomarkers, describe the challenges faced by this research area, and offer suggestions of best practice in order to facilitate the translation of laboratory studies to the clinic in the not too distant future.

ABBREVIATIONS

miRNAs: Micro RNA; ncRNA: Non-Coding (Nc) RNA; FFPE: Formalin-Fixed Paraffin-Embedded; qRT-PCR: Real Time Quantitative PCR; NGS: Next Generation Sequencing; PBMC: Peripheral Blood Mononuclear Cell; rRNA: Ribosomic RNA; snoRNA: Small Nucleolar RNA; dPCR: Digital PCR

INTRODUCTION

MicroRNAs (miRNA) are a class of naturally occurring short non-coding (nc) RNA molecules that regulate eukaryotic gene expression post-transcriptionally. There are over 2500 human microRNAs that have been identified [1,2], and it is believed that more than well over half of all human genes are directly regulated by miRNA [3]. MiRNAs have been shown to play key regulatory roles in nearly every physiological and pathological aspect of biology [4], and there is now vast evidence that dysfunctional expression of miRNAs is a omnipresent feature of many different pathological processes, including cancer [5-7], metabolic disorders [8,9], inflammatory [10,11], cardiovascular [12,13], neuro developmental [14] and autoimmune [15,16] disease, to name but a few. As a consequence there is great interest in the potential clinical use of miRNAs.

The greatest and undoubtedly most immediate clinical potential of miRNAs is them being used as biomarkers. The National Cancer Institute defines a biomarker as "a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process or of a condition or disease". For example, cancer biomarkers qualify as "diagnostic" when they are useful for differential diagnosis, "prognostic" when they help distinguishing between good outcome tumours and bad outcome tumours in the absence of treatment, and "predictive" when they hold the potential of assessing the probability that a patient will benefit from a particular treatment. Indeed, mounting evidence is being produced on the likely usefulness of miRNAs as biomarkers of various diseases [17-24].

The perfect clinically useful biomarker has high specificity, sensitivity and predictive power. MiRNAs do have a number of characteristics that make them attractive candidates as biomarkers when compared to other classes of molecular biomarkers, not least of all their remarkable stability. This feature means that miRNAs not only can be purified from routinely prepared formalin-fixed paraffin-embedded (FFPE) material [5], but that they can also be detected in biological fluids [25]. The vast majority of other RNA classes are degraded by high levels of

RNases present in the blood [26]; however, miRNAs seem to be stable in the blood and are incredibly resistant to fragmentation by either enzymatic or chemical agents [27]. Several studies have used sonication, proteases and detergents to shed light on the mechanism by which miRNAs are resistant to RNase degradation. According to these studies, this stability comes not as a result of chemical modification, but rather because they are protected by their lipid or protein carrier [27-29]. Therefore, miRNA are some of the best candidates to be used as biomarkers, especially in liquid biopsies, which is an increasingly attractive method for sampling patients due to its minimally invasive nature. This great promise faces several challenges: the lack of reproducibility of some results suggests that fulfilling this promise remains a work in progress. Below we discuss some of these issues in more detail.

CHALLENGES, OPPORTUNITIES, AND RECOMMENDATIONS

The circulating miRNome biomarker research field has seen a rapid growth over a very short time (<10 years). However, much of this data associated with particular miRNAs appear to be non-specific, as the same species have been reported in multiple conditions and outcomes. More worryingly, there are great many non-overlapping and even contradictory reports in the literature. The reason for these differences are complex, however the primary cause is biological and technical variation between studies such as the starting material used in experiments (e.g. purification of cells, cell types, control populations used, RNA extraction, etc.), technological platforms (e.g. microarray, real time quantitative PCR (qRT-PCR), next generation sequencing (NGS), etc.), and differing statistical methodologies used. Indeed, the majority of reports regard single-centre retrospective studies using small cohorts, and the standardization of sampling and processing protocols, RNA extraction methods, profiling platforms, and analysis are yet missing.

Sample choice

Although obvious, the choice of starting material is a crucial part of initial experimental design, and the choice of whole blood, peripheral blood mononuclear cells (PBMCs), serum, plasma or purified exosomes from the same individual will generate very different expression profiles [30-32]. Additionally, a systematic investigation into plasma processing conditions showed that processing differences result in variation in platelet contamination in plasma and subsequent significant differences in miRNA abundance [33]. Also, if plasma is the chosen starting material, it should be born in mind that the tubes used for plasma collection contain anticoagulants, including EDTA, heparin or sodium citrate that may interfere in the following downstream applications such as RT-qPCR [34]. Table (1) summarizes the advantages and limitations of measuring miRNA in the most commonly used biological fluids for biomarker discovery.

The blood collection procedure itself is also critical. All of the circulating cell types in blood have their own unique miRNA profiles and given the low concentration of miRNAs in plasma or serum, RNA from a small number of lysed cells can represent a disproportionately large proportion of the miRNAs detected. Cellular miRNA contamination due to lysis can vary from sample to sample if the blood is not collected and processed promptly and carefully, and confound downstream data analysis [35-37]. Therefore, the elapsed time between blood collection and processing should be reduced to prevent lysis and subsequent miRNA contamination [35-39]. Also, it is important to discard the first several ml of blood to prevent contamination from the puncture site [40].

Alternatively, for the study of specific diseases it might be worth considering as starting material body fluids others than blood. Local sampling might be advantageous, before the miRNAs of interest are diluted in the bloodstream or other spaces. For example, levels of 4 miRNAs in the bile have successfully been

Table 1: Summary of advantages and limitations of measuring miRNA in the most commonly used biological fluids for biomarker discovery (modified from [18]).

	Plasma	Serum	Urine
Accesibility	Minimally invasive	Minimally invasive	Non-invasive
miRNA stability	Stable under harsh conditions including boiling, low/high pH, extended storage and multiple freeze-thaw cycles [27,66]	Stable under harsh conditions including boiling, low/high pH, extended storage and multiple freeze-thaw cycles [28,67]	Stable under multiple freeze-thaw cycles [68]
Total RNA quantity	10-300 ng/mL [43]	10-300 ng/mL Conflicting reports: some report lower RNA yield than plasma [30], whereas others report similar yield [69]	1-100 ng/mL [43,70]
	miRNA levels strongly correlate between plasma and serum [27]	miRNA levels strongly correlate between plasma and serum [27]	
PCR inhibitors	Anticoagulants: heparin, citrate		
Interferences with extraction	High protein abundance	High protein abundance	
Cellular contamination	Haemolysis (control: miR-23a and miR-451) Blood cells not separated properly, Cell debris, apoptotic bodies, blood platelets Frequent [37]	Haemolysis (control: miR-23a and miR-451) Cell debris, apoptotic bodies, blood platelets	Urethral cells, cell debris

Abbreviations: RNA; Ribonucleic Acid; RIN: RNA Integrity Number

used to predict acute cellular rejection after liver transplantation [41]; also, miRNA profiling of knee synovial fluid was useful to distinguish early-stage and late-stage knee osteoarthritis patients [42]. Similarly, miRNAs have been detected in other body fluids like saliva, urine, breast milk, cerebrospinal fluid, tears, and vitreous and aqueous humors of the eye, to name a few [18,43].

RNA extraction

Another important source of variability comes from the choice of RNA purification procedure. Importantly, small RNA molecules with low GC content are known to be selectively lost during phenol-based extraction methods (being TRIzol®/ TRI Reagent® the most popular protocols used) when present in low concentrations such as in biological fluids [44]. For that reason, specific commercial kits have been developed for RNA isolation from biological fluids and are widely used. However, most commercially available RNA isolation kits will not capture small RNAs (< 200 nucleotides); fortunately, some companies have developed kits that have been specifically optimized for the isolation of small RNAs species, included in the 10-200 nucleotide range (including miRNAs, 5S rRNA, and U1 snoRNA).

In addition, biological fluids typically contain very high levels of salts, lipids and proteins that can inhibit enzymes used to detect RNA. Many protocols use non-human miRNAs (such as *Caenorhabditis elegans*) miRNAs added to plasma as a spike-in to control for this (and extraction) variability [27]. An additional issue is that it is often impossible to accurately measure RNA in samples from biological fluids and therefore studies frequently use fixed volumes of plasma as a standardization method, assuming that they include samples with certainly different RNA levels [45].

Detection method

Many methods are routinely employed to measure extracellular miRNAs including qRT-PCR (LNA-based, TaqMan or other proprietary technologies), microarrays, next generation sequencing (NGS) techniques, and more recently, digital PCR (dPCR). It is beyond the scope of this mini review to recapitulate the specifics and technical challenges of each of these techniques in detail, which have been substantially covered in other reviews [46,47]. A summary of the features of each these methods is outlined in Table (2). For example, the technique of choice for circulating miRNA discovery is NGS, but the amount of starting material, and the highly specialized personnel and computational infrastructure that it requires for data analysis might make it unfeasible for specific studies. Microarrays could therefore be an attractive alternative for exploring the biomarker potential of known miRNAs, and are widely used, and usually their results have subsequently been validated by qRT-PCR. However, the difficulty in choosing a sound reference gene makes it difficult to robustly interpret the data, as discussed below.

As illustrated, each technique has advantages and disadvantages depending upon the experimental design and resources. What is clear, however, is that the choice of platform greatly influences the end result and a number of reports have shown disparate results from the same sample source using different platforms (e.g. [48,49]).

Normalization strategy

As mentioned above, a challenging issue is the lack of a suitable endogenous reference miRNA or normalization strategy when studying the circulating miRNome in biological fluids. Although global mean normalization is probably the most accurate method for normalization when considering profiling studies, the low number of miRNA species (typically < 100) present in biological fluids makes it unsuitable [50]. Furthermore, even the widely used U6 or U48 in cell-based studies as internal controls, are not present at detectable levels in biological fluids [51-53], and also are known to degrade during storage [54,55]. Alternatively, individual miRNAs are often used such as *miR-16, miR-24* and *miR-425* [25,27,56]; however expression levels of these miRNAs can vary significantly amongst samples depending upon the pathology that is studied [57-59]. An alternative is to use external controls such as miRNAs from *C. elegans* to normalize [27,35,60,61].

In our view, the least variable miRNAs determined empirically for each experiment (using geNorm and/or NormFinder algorithms), an approach taken by some studies [56,59], should be used as normalizer. We understand that this is not always possible when sample volumes are limited. If that is the case, at least two (or preferably three) RNA endogenous controls should be used as standard in circulating transcriptome studies.

CONCLUSION AND FUTURE PERSPECTIVES

In the personalized medicine era we need the tools to be able to efficiently separate patients into different groups, with medical decisions, practices, interventions and/or products being tailored to the individual patient based on their predicted response or risk of disease. In this scenario, biomarkers are needed to make the right decisions at the right time. Liquid biopsies minimize the costs and risks of sampling patients, and allow screening and repeated sampling on patients undergoing therapy. MiRNA are among the best analytes with biomarker potential, mainly due to their stability. Overwhelming evidence points to their potential usefulness as biomarkers in liquid biopsy, but a sustained and systematic effort of the research community for standardizing protocols and reporting of data is still needed to fulfill the biomarker promise of miRNA. Hopefully, the rapid development of technology for detecting miRNA and the reduction of its costs will allow a flourishing of circulating miRNA studies, bringing the field closer to the clinic.

Far from being just a dream, deregulated miRNA are already an attractive target for the development of new therapeutic options in several diseases, and several pharmaceutical companies already have miRNA therapeutics in their developmental pipelines [62]. Moreover, some of them have entered early phase clinical trials, including Miravirsen (SPC3649, Santaris Pharma A/S) for treatment of hepatitis C virus (HCV) infection (NCT01872936, NCT02031133, NCT02508090) [63], miR-16 mimics for treatment of patients with malignant pleural mesothelioma or non-small cell lung cancer (NCT02369198) [64,65], and miR-34 mimics (MRX34, Mirna Therapeutics) for the treatment of several cancer types (NCT01829971). These promising studies highlight the clinical potential of miRNA beyond their biomarker value.

Table 2: Comparison of methods commonly used to study extracellular miRNA. Data analysis: Easy (feasible in any molecular biology lab), Moderate (various software platforms available), Difficult (requires advanced computational infrastructure). Modified from [71].

	Minimum total RNA needed*	Throughput	Ease of customization	Sensitivity	Specificity	Time required to get data	Data analysis	Potential to identify novel sequences
Northern blotting	5 µg	Low	High	Low	High	Days	Easy	NO
qRT-PCR	1 ng	Low	High	High	High	Hours	Easy	NO
Affymetryx GeneChip® miRNA Microarrays	130 ng	High	Low	Low	Low	Days	Moderate	NO
Agilent oligonucleotide miRNA Microarrays	100 ng	High	Moderate	Moderate	Moderate	Days	Moderate	NO
Exiqon miRCURY LNA™ microRNA Arrays	250 ng	High	Moderate	Moderate	Moderate	Days	Moderate	NO
NanoString nCounter® miRNA Expression Assays	100 ng	Moderate	Moderate	Moderate	Moderate	Days	Moderate	NO
Digital PCR	1 copy of target	Moderate	Moderate	High	High	Hours	Easy	NO
Next Generation Sequencing	1 µg	High	High	Moderate	High	Weeks	Difficult	YES

*MicroRNAs constitute only a small fraction (~0.01%) of the total RNA. Attempts to purify this small fraction can result in loss of microRNAs or co-purification of larger RNA species.
Abbreviations: RNA, ribonucleic acid; qRT-PCR, real time quantitative PCR ; PCR, polymerase chain reaction; LNA, locked nucleic acid.

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